Vol. LV. *September, 1928* *No. 3.*

BIOLOGICAL BULLETIN

THE INFLUENCE OF MOLDS ON THE GROWTH OF LUMINOUS BACTERIA IN RELATION TO THE HYDROGEN ION CONCENTRATION, TOGETHER WITH THE DEVELOPMENT OF A SATISFACTORY CULTURE METHOD.

SAMUEL E. HILL,

PHYSIOLOGICAL LABORATORY,

PRINCETON UNIVERSITY.

At the Marine Biological Laboratory, Woods Hole, during August and September, 1927, it was observed that cultures of luminous bacteria (*Bacillus Fischeri, Beijerinck, Migula*) tended to deteriorate rapidly, the deterioration being progressive, so that finally subcultures were made daily, the luminescence becoming steadily less, and the culture was finally lost. A fresh culture was obtained by plating out luminous material of the same stock, recovered from an old Petri dish culture contaminated with mold. This culture grew vigorously for a time, and then degenerated. Since the bacteria in contact with the mold continued to grow and glow for some time, new cultures were isolated when necessary.

These bacteria were being used for physiological experimental material, and it was considered necessary to learn the reason for the deterioration of the cultures and devise a cultural method by which bacteria of the same strain could be maintained in vigorous condition throughout any given series of experiments. Luminous bacteria live normally in sea water, which is maintained constantly in a fairly definite alkaline pH range. They are considered to grow best on culture media of about the same pH value as the sea water. All of these cultures were grown on the same medium, supposedly of the proper pH, and it was sug-

gested by Professor Harvey that the trouble might be due to insufficient alkali reserve, the acid produced by the bacteria rapidly lowering the pH of the medium to a value unfavorable to their growth. The influence of the mold in causing continued light and growth might be due to alkali production. This explanation was favored by the result of pouring a solution of *M/2* NaCl to which Clark's phosphate buffer, pH 8.0, had been added, over the surface of several Petri dish cultures which had ceased to glow. One, in which the light had been out only a few hours, again began to glow, and the luminesceice lasted for over eight hours. Others, in which the light had been extinct for longer periods, were not revived.

Friedberger and Doepner (1907) had studied the influence of various molds on the light intensity of cultures of luminous bacteria. They grew molds in bouillon, filtered the bouillon, and used this material in making up culture media. They found a greater intensity of light in cultures grown on these media than on controls prepared with ordinary bouillon. The one difference which they could establish between ordinary bouillon and bouillon in which mold had been grown was an increased alkalinity in the latter. Their figures show that 10 cc. normal bouillon neutralized .4 cc. *n/*10 NaOH to phenolphthalein, while 10 cc. of their "mold bouillon" neutralized .2 to .4 cc. *n/*10 H_2SO_4 to phenolphthalein. They arrived at the conclusion that the greater intensity of the light of cultures grown on "mold bouillon" was due in part to the increased alkalinity, and in part to "other properties" of the mold.

Molisch (1912) had shown that in general the intensity of light of cultures of luminous bacteria depended on the rate of growth. It is the opinion of the writer, for reasons given below, that the only cause for the increase in intensity of light and length of life of cultures of luminous bacteria grown in contact with mold is that of alkali production by the mold, which thus acts as an alkali reserve.

A series of experiments using solutions of *M/2* NaCl plus Clark's phthalate, phosphate, and borate buffers, found to be nontoxic, showed that these bacteria glowed brightly in the pH range 5.7 to 8.7, the luminescence lasting for over an hour.

(Observations were not made after more than an hour had elapsed.) Below pH 5.2 the light lasted only a few seconds, above pH 9.0 for three minutes or less. The pH range in which growth can be expected lies then between 5.7 and 8.7, pH values outside this range being productive of rapid injury.

The culture medium in use was a peptone, beef-extract, glycerine agar, made up in sea water, the pH being adjusted to 8.2 with NaOH. As these bacteria live normally in an environment containing NaCl in about one half molecular concentration, favorable conditions are provided for the use of buffer mixtures. Molisch (1912) had shown that a number of salts other than NaCl might be used in culture media for luminus bacteria. A culture medium was made up in which one fifth mol of secondary potassium phosphate in 500 cc. distilled water was substituted for one half of the sea water. After sterilization the pH was adjusted with NaOH to 8.2. Separate lots of the same batch were colored with the Clark and Lubs selection of indicator dyes, covering the pH range from 1.2 to 9.8. Cultures were started on slants prepared from these media, six tubes of each being inoculated with luminous bacteria and three of each six being inoculated also with a common mold at one end of the slant. (The mold used was kindly identified for me by Dr. Charles Thom, as *Penicillium* sp., in the same section with *P. commune* (Thom) and *P. solitum* (Westling).) These were all allowed to develop somewhat below room temperature for two weeks.

Some of the indicators used were accumulated by the bacteria. These are being studied further to determine whether they penetrated the cell, or were merely adsorbed on the surface. They were of little value for this study, since not enough dye was left in the medium to indicate its pH value. However, in the case of several of these, the pH was indicated roughly by the color of the dead bacteria, which was not markedly different from the medium. With brom cresol green (yellow at 3.8, blue at 5.8), the dead bacteria near the mold were a more intense blue than elsewhere, and the acid range of the indicator had not been reached anywhere in the slant.

On the chlor-phenol red slants (yellow at 5.2, red at 6.8) the color of the medium indicated that the pH had been reduced to

5.4 ∓ .2. The pH of the medium near the mold was well above the alkaline range of the indicator.

On the cresol red slants (yellow at 7.2, red at 8.8) the color of the medium indicated pH below the range of the indicator except near the mold, where a pH of 8.6 ∓ .2 was indicated. The results with meta cresol purple were about the same. With thymol blue, the color of the indicator was masked by the color of the medium at the critical value, and it was of no value.

On the same date six cultures were started on medium of the same batch without addition of indicator. At the end of two weeks all were alive and glowing brightly. These cultures decreased slowly in brilliance during the next month, but were still glowing faintly at the end of six weeks, and viable transfers were made at the end of the seventh week. The final death of these cultures appeared to be caused by the drying up of the medium.

As a further check on the alkali influence, several cc. of *M*/NaOH was introduced at the bottom of each of six slants of unbuffered medium colored with brom thymol blue, and an equal number without indicator. Streaks made on these slants developed rapidly on the upper half of the slant, away from the alkali, and grew well, the cultures on the uncolored medium lasting for several weeks (average of six, 22.2 days), until the alkali was exhausted. On one of these, more alkali was added and a fresh inoculation made, the growth lasting this time for less than a week. It was observed that no growth took place below the line which marked the limit of diffusion of strongly alkaline NaOH. This limit was well marked on the uncolored medium by the precipitation of magnesium hydroxide.

The most characteristic activity of luminous bacteria seems to be that of acid production. They are killed in a few days in their own acid if some method of neutralization or removal is not employed. In their natural environment the excess acid would simply diffuse into the surrounding sea water, but within the limits of the test tube this cannot occur. The base used in the culture medium was NaOH, which in contact with carbon dioxide becomes $NaHCO_3$, and since $NaHCO_3$ in the concentration used (.01 *M*) is not particularly acid when saturated with carbon dioxide, it is not likely that the acid limiting their growth is car-

bon dioxide. That it is a non-volatile acid is shown by the following experiment:

A constant stream of sterile air was drawn in series through three bottles of slightly buffered culture medium colored with cresol red. The first of these was the control, without bacteria. The other two were inoculated with luminous bacteria. At the end of 24 hours the control was red, as at the start, and uncontaminated as shown by the absence of turbidity, and this condition lasted until the close of the experiment. The two inoculated bottles at the end of 24 hours were down to about pH 7.4. Enough NaOH was added to the third bottle to restore the original pH of approximately 8.2, and this was repeated every two hours until the close of the experiment. At the end of 36 hours, the PH in bottle No. 2 was down to about 5.5 (determined by withdrawing some of the material and testing with other indicators) and the light was extinct. In bottle No. 3, in which pH 8.2 was maintained, the bacteria continued to glow for another 24 hours, when the light failed, due presumably to failure of the food supply. Carbon dioxide and any other acids volatile at room temperature (if any were formed) would have been swept out by the stream of air, leaving behind the non-volatile acid. This is probably lactic acid.

Other culture media were tried in which calcium and barium carbonates were employed as buffers, and also higher concentrations of K_2HPO_4 and sea water, and lower concentrations. Luminous bacteria can tolerate a considerable range of salt concentration. It was found that on phosphate buffered media where the total salt concentration was greater than in sea water, but not in excess of molar concentration, growth was slower than on media of the proper concentration, and the tendency to diffuse growth was absent. The resultant crowding gave the streaks a fictitious brilliance for a few days, after which the light intensity decreased to a low value. These cultures were viable for fairly long periods of time, average 21 days, but not as long as cultures on media of the proper salt concentration. When media of lower total salt concentration (as about ¼ molar) were used, there was an initial rapid growth, accompanied by flowing over the surface of the medium, and a rapid decay, so that such cultures were

viable for only a few days, the average of six cultures being five days. Since a heavy precipitate of calcium and magnesium phosphates was formed when the phosphate buffer was added to sea water, media were prepared containing various concentrations of NaCl, from .25 *M* to .75 *M*, as substitutes for sea water, but these were unsatisfactory, the best of them lasting for only 14 days.

On medium buffered with calcium carbonate, growth was vigorous, but the life of the cultures was less than with the best of the phosphate buffer mixtures. The average length of life of eleven cultures without indicator was 17.8 days. Curiously enough, the death of these cultures was due to excess alkalinity. The initial growth was rapid, but on the third or fourth day there was a decrease in brilliance of light and a slowing down of growth, caused by the rapid diffusion of the acid through the agar, using up the small amount of calcium hydroxide in solution. This was followed by an increased brilliance and renewed growth as the pH rose again, due to the solution of more calcium hydroxide (produced by hydrolysis from the calcium carbonate), and its diffusion through the medium. The calcium salt of the acid produced by the bacteria is much more soluble than calcium carbonate, and is evidently hydrolyzed in solution, for the medium becomes steadily more alkaline until the alkaline range of the available indicators is passed. Since the bacteria are soon killed by alkali above pH 9.0, the limiting value is passed, and luminescence ceases. This can happen only when the calcium carbonate is in excess. When the pH of the medium was adjusted with calcium carbonate, and the excess carbonate filtered off, initial growth was rapid, but the decline following it continued until the death of the culture occurred on the sixth day (average of six cultures), caused by acidity as shown by the use of a suitable indicator.

On the medium prepared with barium carbonate from which the excess carbonate was filtered off, the initial fair growth was followed by a rapid decline, the average length of life of 14 such cultures being 6.5 days. When an excess of barium carbonate was present, the initial growth was fair, and slowly decreased, the cultures growing steadily more alkaline, the average length

of life of 14 cultures being 17.5 days. Although theoretically about the same pH value should be produced by barium and calcium carbonates, in practice the medium prepared with barium carbonate was always the more alkaline, and was too alkaline for good growth of the bacteria. The vigorous growth obtained on calcium carbonate was never obtained on media prepared with barium carbonate.

The medium prepared with calcium carbonate has the advantage that no adjustment of pH is required, the hydrolysis of the carbonate giving approximately the right value. It is by far the best buffer substance to use, both for slants and for Petri dish cultures. The medium should contain 20 grams "Bacto" nutrient agar, 10 cc. glycerine, and 5 grams calcium carbonate per liter, made up in sea water. If a transparent medium is desired, the phosphate buffer mixture with the same amount of nutrient substance, made up in sea water and filtered, may be used. The optimum pH value for this medium, probably about 8.6, may be secured by titrating the hot medium by the drop method until a good red is secured with cresol red, and a barely perceptible color with thymol blue. When one fifth mol of buffer is added to sea water, the average life of cultures emitting strong light is 18 days. After this time, very little light is emitted, but viable transfers may be made for several weeks.

Of the indicators used, several appeared to be slightly toxic to the bacteria, but the evidence on this point is inconclusive.

Summary.

The influence of molds on the length of life of cultures of luminous bacteria may be simulated by the use of buffer mixtures, or by supplying fresh alkali continually. The maximum alkalinity produced in these experiments by the influence of *Penicillium* sp. was pH $8.6 \mp .2$. Degeneration of cultures of luminous bacteria may be caused by growth on media insufficiently alkaline, or so slightly buffered that it soon becomes acid. Diffuse growth and spreading over the surface of the slant is caused by too low salt concentration. Long life of cultures may be secured by growing on media sufficiently alkaline, and sufficiently buffered to resist rapid change by the acid production of the bacteria, which are killed by their own acid at about pH 5.6.

BIBLIOGRAPHY.

Clark, W. M.
'27 The Determination of Hydrogen Ions. Baltimore.
Fischer, B.
'88 Ueber einen neuen lichtentwickelnden Bacillus, Centralbl. f. Bakt., etc., 3, Nos. 4 and 5.
Friedberger, E., and Doepner, H.
'07 Ueber den Einfluss von Schimmelpilzen auf die Lichtintensität in Leuchtbacterien-culturen, etc., Centralbl. f. Bakt., etc., 1st Abt., 43, 1.
Migula.
'00 System der Bakterien Zweiter Band, Jena.
Molisch, Hans.
'12 Leuchtende Pflanzen, eine physiologische Studie, Zweite Auflage, Jena, 1912.